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# Variations in Human Serum Lipoprotein Detected by Isoelectric Focusing

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Following the method of Kostner and coworkers with some modifications, human serum lipoproteins of native serum and isolated fractions were studied by means of isoelectric focusing with ampholine 3—10 (LKB Instruments). Lipoprotein fractions were prepared by sequential preparative ultracentrifugation at increasing densities. Sera and fractions from 10 healthy donors were repeatedly investigated to evaluate possible environmental factors on the expression of banding patterns. As a result, at least two of the observed bands seem to vary depending on whether the sera were obtained from fasting or nonfasting individuals. The investigation of 158 related individuals yielded some insight into a possible genetic determination of specific bands. It may however be difficult to distinguish all genetic and environmental factors involved. Isoelectric focusing may soon be of great clinical value in the diagnosis of type I to V hyperlipoproteinemia.

In Anlehnung an die Methode von Kostner und Mitarbeitern wurden menschliche Serumlipoproteine in Vollserum und isolierten Fraktionen mit Hilfe der isoelektrischen Fokussierung unter Verwendung von Ampholine 3—10 der LKB Instruments untersucht. Die Lipoproteinfraktionen wurden durch schrittweise präparative Ultrazentrifugierung in Medien aufsteigender Salzdichte gewonnen. Um umweltsbedingte Faktoren in der Ausprägung der Bandenmuster zu erkennen, wurden zehn gesunde Spender wiederholt untersucht. Tatsächlich scheinen sich wenigstens zwei Banden unterschiedlich darzustellen, je nachdem, ob das Serum von der Person nüchtern oder nichtnüchtern entnommen wurde. Die Untersuchung von 158 verwandten Individuen erbrachte Hinweise auf eine mögliche genetische Steuerung einzelner Banden und ihrer Ausprägung. Im Hinblick auf das Zusammenwirken von genetischen und nichtgenetischen Faktoren müssen diese Ergebnisse jedoch mit Zurückhaltung interpretiert werden. Von großem Nutzen dürfte diese neue Technik jedoch bei der Diagnose der verschiedenen Typen der Hyperlipoproteinämie nach Fredrickson sein.

In the last two years, a systematic search for individual variations in human serum lipoproteins has been undertaken, applying combined preparative and electrophoretic methods. As a result, the El (C) system was detected in a lipoprotein fraction of density $1.063 < \varrho < 1.1$ g/ml (1). Another electrophoretic variation — the El (BA) types — of this lipoprotein fraction were the subject of a recent paper (2).

To extend this program to new analytical methods, we also applied isoelectric focusing to the study of the lipoproteins of complete serum, and to isolated lipoprotein fractions. Using a mixture of ampholytes with a range of isoelectric points in a stabilizing medium to prevent convective disturbance, it is possible to separate proteins according to their specific isoelectric points by applying an electric field (3). The application of this method to the study of lipoproteins was greatly hampered by aggregation of the proteins until Kostner, Albert and Holasek (4) introduced ethylene glycol as a stabilizing reagent for the focusing process. It should be stressed that the principle of separation is quite different from the usual discelectrophoretic methods, insofar as the polyacrylamide gel serves merely as a supporting medium, not functioning as a molecular sieve, while the lipoprotein fractions are separated solely according to their pI. The resolving power of this method is generally accepted to be excellent: proteins differing in isoelectric point by less than 0.1 pH units could be resolved by isoelectric focusing (5).

This communication is a preliminary account of the results of our study of human serum lipoproteins. In contrast to Kostner and coworkers we were also able to study isolated lipoprotein fractions, with no indication of precipitation or aggregation. In healthy people, the influence of the nutritional state on the expression of banding patterns was investigated. In addition, an attempt was made to distinguish between environmental and possible genetic factors, responsible for the observed variation of phenotypes.

## Material and Methods

Blood was obtained from fasting and nonfasting individuals. The serum and the isolated fractions were studied by isoelectric focusing following the method described by Kostner and his colleagues with some modifications. In addition to repeated investigations of the serum lipoprotein of ten healthy donors, a series of 31 families with 96 children was studied. First, serum lipoproteins were prepared as reported previously (1) removing VLDL, LDL and HDL[2]) by a sequential preparative ultracentrifugation at increasing densities, adding KBr/NaCl solution according to the formula given by Havel, Eder and Bragdon (6). After dialysis overnight against several changes of physiological saline, volumes of 30 to 50 $\mu$l complete serum and 0.1 ml of 1—2% solutions of isolated lipoproteins, resp., were prestained with one to three drops of a freshly prepared saturated solution of

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[2]) Abbreviations: VLDL = Very low density lipoproteins, LDL = Low density lipoproteins, HDL = High density lipoproteins, pI = isoelectric point. El = Electrophoretically detectable lipoprotein variants.

sudan black in ethylene glycol, according to McDonald and Ribeiro (7). These volumes were mixed with appropriate volumes of a solution of the following composition: 2.5 g acrylamide, 0.1 g bisacrylamide, 2.5 µl N.N.N'.N'.-tetramethylethylenediamine, 2.5 ml 40% ampholine 3—10 (LKB carrier ampholytes), 0.5 mg riboflavin, 19 ml ethyleneglycol and dist. water ad 50 ml. For the fractionation, a discelectrophoretic apparatus of Shandon Comp. was used. Throughout this investigation, separations were performed in tubes of 8 mm inner diameter and 80 mm long. 5% phosphoric acid and 5% ethylendiamine were placed in the upper and lower buffer compartments, resp. The initial voltage was adjusted to approx. 80 V not exceeding 2 mA per tube. After one to two hours the voltage can be raised to 140 V per run. Under these conditions, electrophoresis was usually stopped after 6 to 8 hours. In the beginning, air bubble formation severely handicaped our study. This was overcome by carefully controling the quality of dist. water used and by raising the concentration of ethyleneglycol to 40%.

The focusing process usually was stopped at a point when all the bands had formed as single sharp lines easily distinguishable from each other. This point was nearly but not fully identical with the end point of the focusing process where the bands may be focused so closely together that individual patterns may hardly be visible. Immediately after stopping the electrophoresis, photographs and densitometer curves were taken of each gel, and the patterns were also drawn on graph paper. In a number of gels, the pH of individual bands was measured with a pH meter type 390 from WTW Werkstätten, Weilheim, using a microelectrode No. LoT 406-M 3 from Dr. Ingold, Frankfurt/Main, inserting the electrode directly into the gel after its removal from the glass tube.

## Results

In some preliminary experiments, the influence of different batches and concentrations of ampholine, stain, ethyleneglycol and time of electrophoresis was evaluated. Slight differences were noted between different batches of ampholine leading to minor changes in the sharpness of individual bands and their distance from neighbouring bands. Further, different lipoprotein bands may have slightly differing avidities for the lipid stain, so that undersaturation could result in an unreliable expression of the minor bands. The overall reproducibility of this technique, however, was excellent.

The types of patterns observed in complete human serum are depicted in Figure 1. For better orientation, the major bands were numbered with Roman numbers. As can be seen, the main dye intensity is centered in region IV and V. Obviously, both regions may consist of two or more sharp bands indistinguishable after formation of the pH gradient. The localization of the ten clearly visible bands may be seen in Figure 3. As judged from the study of approximately 200 individuals of different age, the VLDL of density $\varrho < 1.006$ appear as a mixture of portions of LDL and HDL. The LDL mainly comprise the lower part of the pattern from bands V to X; $HDL_2$ and $HDL_3$ as well as lipoproteins of density $1.063 < \varrho < 1.1$ g/ml, however, exclusively exhibit bands in regions I to IV.

The significance of band I remained obscure: even in 24 hour-runs, after termination of the focusing process, the pH of this band was found to be 3.5 to 3.7. Obviously, this band is most prominently expressed in individuals of strong Lp(a+) type (8). The pI of Lp(a) lipoprotein in contrast was found to be 4.9 when measured in a highly purified preparation (9). Since we were not yet able to obtain a specific precipitation line applying Laurell's technique, the identity of this band remains to be determined. While band II is regularly seen as a double band in all sera, variation was noted in band III. Figure 4 may document an interrelationship between band III and VII: band III appears to be better visible in sera from fasting individuals, compared to the nonfasting condition in the same individuals.

Fig. 1
Isoelectric focusing patterns of human serum lipoproteins from complete serum, from the left: father, mother and their three children (F 24/71)

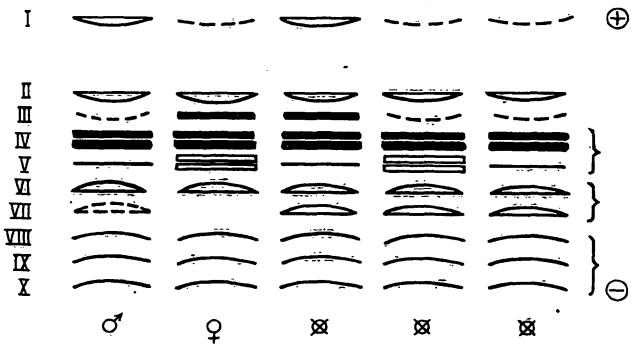


Fig. 2
Schematic representation of Figure 1

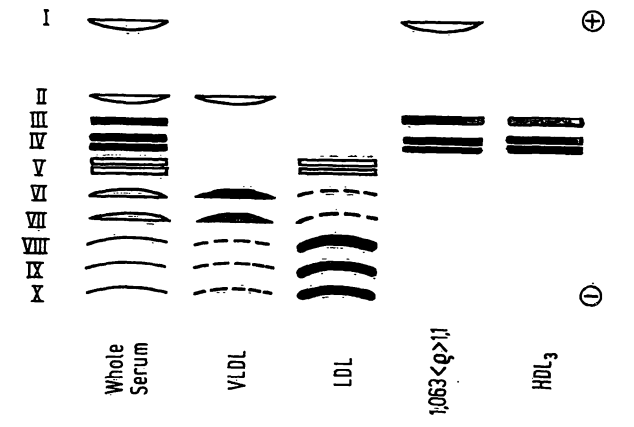


Fig. 3
Schematic representation of iso-focusing patterns of isolated lipoproteins compared to whole serum

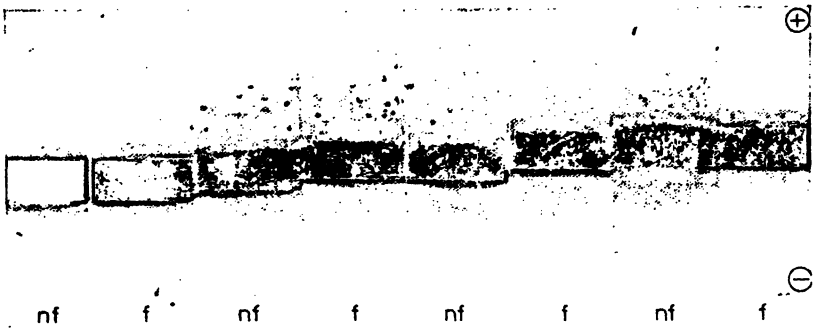


Fig. 4

Interrelationship between bands III and VII as judged from the comparison of fasting and non fasting serum samples from the same individuals. f = fasting, nf = non fasting

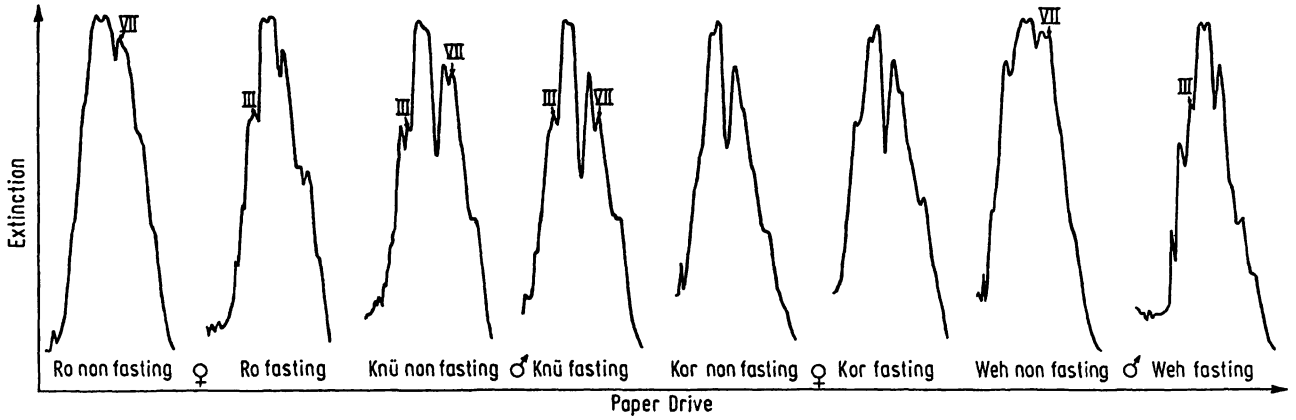


Fig. 5

Densitometer curves of samples from Figure 4. Note the disappearance of bands VII in fasting individuals Ro and Weh, the decrease in individual Knü and no change in individual Kor

Simultaneously an increase in intensity of band VII can be observed in the nonfasting state. It may also be noted (see Fig. 2) that bands VI and VII are the main bands in the very low density lipoproteins of density $\varrho < 1.006$. It is however not clear whether the nutritional state is the only reason for this variation. It could also be influenced by other environmental factors such as diet etc. The switch from a pronounced band III and absent VII to a weaker III with strong VII appeared also to be better visible in men than in women. Densitometer curves of the patterns in Figure 4 are depicted in Figure 5. Regions IV and V are of a rather complex nature. A variation in the relative intensity of these regions could be observed, possibly representing three types: Type A with a leading region IV, type C with a leading region V, and type B with the two regions in about equal intensity. The minimum number of single lines may be 5 to 6. Figure 1 showing the sera of a family — father, mother and three of their children — raises the question as to whether this variation is under genetic influence: two offspring exhibit patterns very similar to that of the father, while one child looks like the mother. This question cannot yet be definitely answered, since many factors may influence the expression of a banding pattern in a given individual. As shown in Figure 6, the number of visible bands is clearly related to the volume applied — two sera were fractionated with 30, 60 and 90 $\mu$l each.

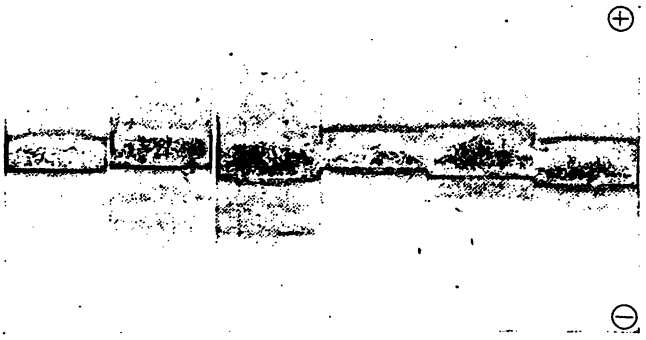

Fig. 6

Application of 30, 60 and 90 $\mu$l, compared in two samples. Note appearance of band III in the second tube and region V band (s) in tubes 5 and 6

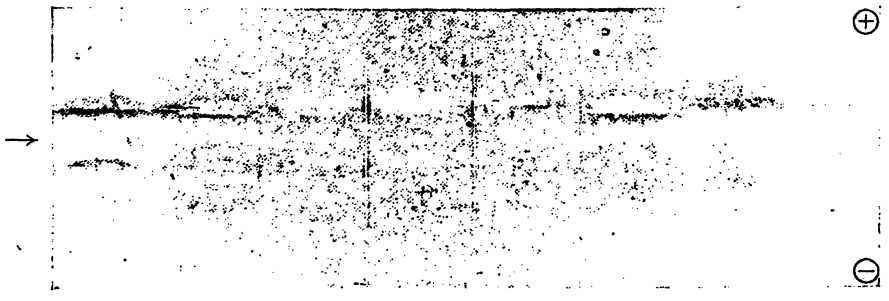

Fig. 7

Isolated $LDL_1$ and $LDL_2$ fractions from eight different individuals

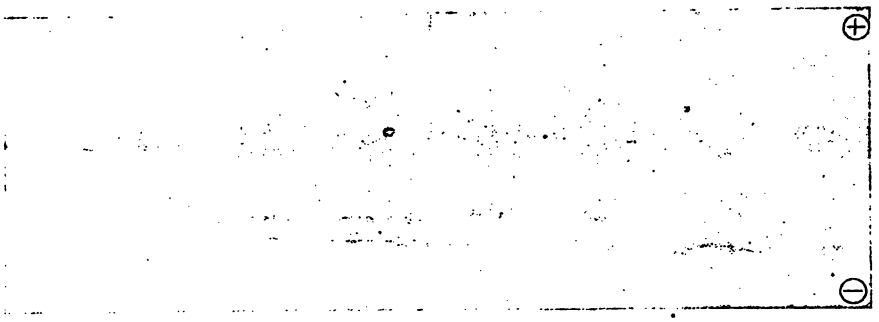

Fig. 8

Isolated VLDL fractions from eight different individuals

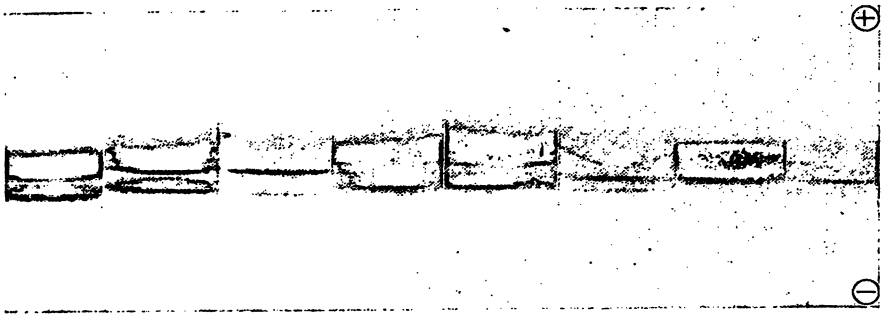

Fig. 9

Iso-focusing patterns of isolated lipoproteins floating between 1.063 and 1.1 g/ml from eight different individuals

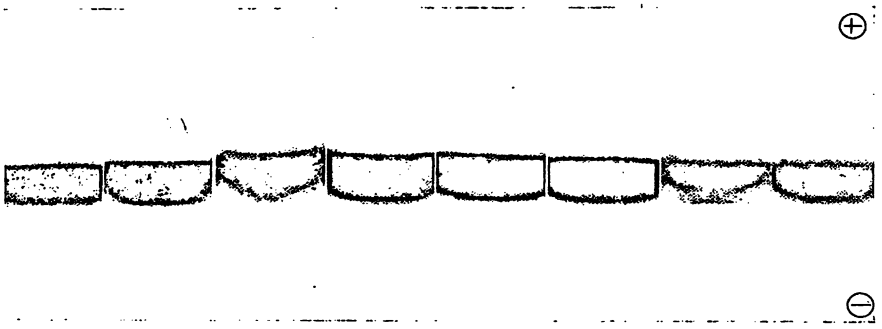

Fig. 10

Isolated $HDL_2$ and $HDL_3$ after isoelectric focusing

As mentionned above, the undersaturation with sudan black can be another source of variation. Finally, band IV and V patterns also varied depending on whether 1% or 5% was chosen as the concentration of phosphoric acid and ethylenediamine, resp.

Another variation was observed when LDL preparations of individuals of different ages (i. e. family members) were compared: As seen in Figure 7, some adults seem to possess an additional band in the VI/VII region (see arrow). It could however not be excluded that this band was in fact a trace of VLDL VI/VII bands not completely removed in the lower density class (see Fig. 8).

Slight variation was also noted in the class of $1.063 < \varrho < 1.1$ g/ml lipoproteins. As reported in a recent paper (2) this is a rather heterogeneous class containing the electrophoretically detectable lipoprotein variants El (C) and El (BA) in addition to $HDL_2$ lipoproteins. In order to distinguish the lines in region IV, the focusing process has been stopped just before termination in Figure 9. Broad black bands can be attributed to $HDL_2$ and $HDL_3$ (Fig. 10). Concluding from these patterns, the El (C) and El (BA) variations seem not to depend on molecular substitutions leading to wider pI changes in the lipoprotein molecule.

Generally, the number of bands observed decreases with increasing lipoprotein density. With the exception of band I, bands II to X were found within the pH range of 4.8 to 5.5 after termination of the focusing process.

## Discussion

Isoelectric focusing provides a new approach to the diversity of human serum lipoproteins. Compared with conventional electrophoretic methods, including disc-electrophoresis, this technique produces by far the largest number of distinguishable bands.

The reproducibility of the method is excellent provided the working conditions are carefully controled. Not only complete serum, but isolated lipoprotein can also be fractionated without evidence of aggregation.

The aim of this study was an attempt to distinguish between quantitative variation and possible genetic factors governing lipoprotein fractions of different pI. Some facts should be kept in mind when approaching this problem. Variation of expression of bands may point to very different things. First, the nutritional state may influence a given line as shown for the bands III and VII. This is not surprising since band VII is a component of the VLDL. Second, if a band seems to be lacking in a given serum, it is hard to show that the corresponding band is really absent. It is even more difficult to prove that a given variation is due to a

genetically determined quantitative variation as shown by the controversy over Lp(a) (10—12). Third, technical factors may be responsible for the different expression of bands as demonstrated for the variation of region IV and V. Fourth, even if it is concluded that a certain variation could be genetic — because of striking similarities between patterns of related individuals — the interpretation could still be invalid suggesting a monofactorial inheritance. This is a situation often met in the genetics of hyperlipoproteinemias (13). Fifth, if the variation is genetically determined, a more complex analysis could be necessary when certain bands appear to be interrelated.

From these considerations, it is concluded that polymorphism of human serum lipoproteins based on distinct pI differences was not unequivocally demonstrated by this study. Further work will be necessary to evaluate all the factors that may interfer with an assumed genetic determination of a given banding pattern.

The most important application of this technique, however, could be seen in the clinical diagnosis of the different types of hyperliproteinemia (13):

1) Isoelectric focusing achieves the best discrimination between components of the different lipoprotein classes in complete serum.

2) The reproducility of the method is excellent, and the quantitative evaluation of single components can easily be performed.

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